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# Adhesion formation assay

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An abbreviated version of this protocol was published in eLIFE in Jan 2022

Synergistic phase separation of two pathways promotes integrin clustering and nascent adhesion formation

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## Detailed protocol

**Reagents**

Fibronectin (Sigma Aldrich, F1141-2MG)
Paraformaldehyde (Fisher Scientific, AA433689M)
Mouse monoclonal anti-Paxillin (Clone 349/Paxillin) (BD Biosciences, Cat#610051; RRID: AB_397463)
Goat polyclonal anti-Mouse IgG, Alexa Fluor 568 (Thermo Fisher, Cat#A-11004; RRID: AB_2534072)
Vectashield (Fischer Scientific, H1000NB)

**Buffers**

Cytoskeleton Buffer:
10mM MES pH 6.1
138 mM KCl
3mM MgCl
2mM EGTA
Sterile filter 0.4um and store at 4 degrees

TBS:
20 mM Tris HCl pH 7.4
150 mM NaCl

TBS-T:
TBS + 0.1% Tween 20

Permeablization buffer:
Cytoskeleton buffer + 0.5% triton-X

Quenching buffer:
Cytoskeleton buffer + 0.1 M glycine

Block:
2% BSA in TBS-T (0.2g in 10mL)

Prior to beginning experiment, coat glass coverslips (#1.5 glass) with fibronectin:

1. Prepare a solution of 10 µg/mL fibronectin in PBS.
2. Cover the glass surface with the solution
3. Incubate overnight at 4 deg.
4. Wash three times with 1X PBS prior to plating cells.

**Cell Adhesion Assay**

1. Detach cells from dish with trypsin.
2. Gently pellet cells and resuspend in media.
3. Plate cells onto fibronectin coated coverslips. Immediately start a timer. You may need to optimize the number of cells to plate in your dish.
    a. For a 5-min time point, unbound cells were gently washed away with fresh media after 1 min. To wash the cells, aspirate the media, carefully add fresh media. Gently swirl the plate, aspirate media, and gently add fresh media. Then incubate at 37°C for another 4 min (total spreading time of 5 min)

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b. For a 20-min time point, unbound cells were gently washed away with fresh media after 10 min. To wash the cells, aspirate the media, carefully add fresh media. Gently swirl the plate, aspirate media, and gently add fresh media. Then incubate at 37°C for another 10 min (total spreading time of 20 min).

4. After the full spreading time (either 5 min, or 20 min) fix cells with 3% paraformaldehyde prepared in Cytoskeleton Buffer. Incubate in fixative for 20 min at room temperature.
5. Permeabilized cells for 8 min with Cytoskeleton buffer + 0.5% triton-X (Permeabilization buffer).
6. To quench the paraformaldehyde, add Cytoskeleton buffer + 0.1 M glycine (Quenching buffer) and incubate for 10 min. 1M glycine stock solution should be prepared fresh immediately prior to experiment.
7. Rinse cells 2 × 5 min in TBS-T.
8. Block cells with 2% BSA in TBS-T (blocking buffer) for 1 hr at room temperature.
9. Add primary antibody (Ms anti Paxillin, 1:100 prepared in blocking buffer) Incubate overnight at 4 °C.
10. The following day, rinse 3 × 5 min in TBS-T.
11. Add secondary antibody (anti Ms Alexa568, 1:250 prepared in blocking buffer). Incubate for 45 min at room temperature. Once you add the fluorescent secondary antibodies, protect samples from light by covering in aluminum foil.
12. Rinse 3 × 5 min with TBS-T.
13. Prepare samples for microscopy with 561 illumination (i.e.Rhodamine/Cy3 settings). Alternatively, you could use different secondary antibodies to be compatible with other wavelengths.
    a. If you are imaging cells with TIRF microscopy, remove TBS-T and add PBS. Mounting media containing glycerol is not ideal for TIRF microscopy.
    b. If you are imaging cells with confocal or epifluorescence, samples can be prepared with mounting media to reduce photobleaching. We mount coverslips on slides using Vectashield.

**Counting Adhesions in ImageJ:**

1. If you are overexpressing proteins in your experiment, we recommend deciding on a narrow range of overexpression levels to image and quantify. To reduce experimental noise due to differences in expression levels, we only analyzed GFP-transfected cells if the mean GFP intensity within the cell fell within a defined range (intensity between 1000 and 5000 a.u. following background subtraction, at least 50% of imaged cells were retained with these cutoffs).
2. Adhesions were segmented and counted with ImageJ macros based on a previously published ImageJ workflow (Horzum et al., 2014).
    a. First macro: run("Subtract Background...", "rolling = 50 sliding"); run("Enhance Local Contrast (CLAHE)", "blocksize = 19 histogram = 256 maximum = 6 mask=*None* fast_(less_accurate)"); run("Exp"); run("Enhance Contrast", "saturated = 0.35");
    b. Then manually run LoG 3D plugin with sigma = 2.
    c. Final macro: setAutoThreshold("Default dark"); setOption("BlackBackground", false); run("Convert to Mask"); run("Invert"); run("Analyze Particles...", "size = 5–1000 circularity = 0.00–1.00 summarize").
    d. The final mask was compared to the original image to visually confirm the results. If the mask doesn't look like it is accurately segmenting focal adhesions, you may need to change some of the parameters in the macros.
3. We also validated the final results of automated analysis by manually segmenting adhesions (data not shown).

**How to cite:**(Readers should cite both the Bio-protocol preprint and the original research article where this protocol was used)

1. Case, L. and Rosen, M. (2022). Adhesion formation assay. Bio-protocol Preprint. bio-protocol.org/prep1918.
2. Case, L. B., De Pasquale, M., Henry, L. and Rosen, M. K.(2022). Synergistic phase separation of two pathways promotes integrin clustering and nascent adhesion formation. eLIFE. DOI: 10.7554/eLife.72588